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## The role of drug efflux pumps in acute myeloid leukemia

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## SUMMARY

Acute myeloid leukemia (AML) is a malignant clonal disorder, characterized by the accumulation of an immature cell population in the bone marrow. The expansion of the leukemic clone eventually results in suppression of the normal hematopoiesis. Treatment with intensive chemotherapy is the only option to eliminate the malignant cells from the bone marrow. Although many patients achieve a complete remission after intensive chemotherapy, a number of patients are refractory to this treatment, and a large proportion of the patients shows a relapse after having reached complete remission, illustrating treatment failure. Different prognostic factors have been defined that are predictive for treatment outcome in AML<sup>1</sup>. Low white blood cell counts, structural abnormalities in karyotype, such as inversion(16), translocations(16;16), (15;17) and (8;21), are important factors determining good response. Other factors, including high white blood cell counts, immature immunophenotype, increasing age, structural karyotypic abnormalities such as a deletion of chromosome 5 or 7, and an internal tandem duplication of the *Fli3* gene on chromosome 13<sup>2,3</sup>, are associated with a worse outcome in AML.

In addition, treatment failure in AML is associated with the presence of intrinsic or acquired resistance to chemotherapeutic agents. One type of drug resistance mechanisms is characterized by the resistance to several structurally and functionally unrelated drugs, and is called multidrug resistance (MDR). The overexpression of adenosine triphosphate (ATP)-dependent membrane transporter proteins, which function as drug efflux pumps<sup>4</sup>, has been identified as a MDR mechanism. Transfection and overexpression studies with the genes encoding drug efflux pumps in human cell lines have shown that these transporter proteins confer resistance to a wide range of chemotherapeutic agents, including daunorubicin, doxorubicin, mitoxantrone and etoposide, which are drugs used in the treatment of patients with AML.

The studies described in this thesis are focused on the role of the drug efflux pumps P-glycoprotein (P-gp), encoded by the *MDR1* gene, the multidrug resistance protein 1 (MRP1) and its homologues, especially MRP2, MRP3, MRP5 and MRP6, and the novel breast cancer resistance protein (BCRP)<sup>5</sup>, in AML.

In **chapter 1** an overview is given of the current knowledge concerning these transporter proteins, with emphasis on the expression and especially the functional activity of the drug efflux pumps in AML cells. P-gp has been shown to be associated with poor

treatment outcome in AML patients. Besides P-gp, MRP1 appeared to contribute to the observed resistance in AML. MRP2, MRP3, MRP5, MRP6 and BCRP, have been shown to be expressed at variable levels in AML patient cells. The latter proteins have been described to confer resistance to chemotherapeutic drugs such as daunorubicin, mitoxantrone, etoposide and 6-mercaptopurine, which are generally used in the treatment of AML patients; however, they have not yet proven to play a role in drug resistance in AML.

**Chapter 2** describes the development of a functional assay to measure the activity of MRP1. The fluorescent compound carboxyfluorescein, which is formed after cleavage of the non-fluorescent carboxyfluorescein diacetate by intracellular esterases, and which is transported by MRP1, was used in a flow cytometric assay. In addition it was shown that the transport of carboxyfluorescein could be inhibited by the leukotriene D4 receptor antagonist MK-571 in several MRP1 overexpressing cell lines. Subsequently, the MRP activity was measured in 15 AML samples, and appeared to vary widely between the cases. The MRP activity correlated positively with MRP1 protein expression. Besides MRP1 protein expression, mRNA transcripts of the MRP1 homologue MRP2 were demonstrated in 40% of the AML cases.

In this study 2 patients with inversion of chromosome 16, on which *MRP1* is located, were included. These patients showed a low MRP activity, possibly reflecting the dysfunction of the MRP1 pump as result of the chromosomal inversion. This stimulated the study in **chapter 3**, where we focussed on AML patients with inversion(16). In a paper by Kuss *et al.*<sup>6</sup>, it has been reported that a deletion of one *MRP1* allele in patients with inversion(16) was associated with a prolonged duration of disease free survival, suggesting an important role for MRP1 in determining clinical outcome. That study did not evaluate the MRP activity in this patient group. We therefore analyzed *MRP1* deletion by fluorescent *in situ* hybridization (FISH), with a focus on inversion(16) patients, and found a deletion of 1 or 2 *MRP1* alleles in 7 out of 11 patient samples. The occurrence of these deletions correlated with low MRP activity. However, the MRP activity in the AML cases was not different from that in normal hematopoietic cells. Furthermore, the mRNA expression of MDR1, MRP1, MRP2 and MRP6 were determined, and MRP2 and MRP6 mRNA appeared to be predominantly expressed in AML samples with 1 *MRP1* allele, whereas in normal bone marrow cells no MRP2 and MRP6 mRNA was observed. In addition, P-gp activity was observed in a patient sample which showed no MRP activity and no expression of MRP mRNA transcripts. In conclusion, these findings demonstrate that in AML patients with inversion(16) no

distinct difference in MRP activity can be observed compared to normal hematopoietic cells, despite the deletion of 1 or 2 *MRP1* alleles. This could in part be due to the upregulation of other MDR genes. Therefore, the favorable prognosis of inversion(16) AML patients is not necessarily caused by the deletion of *MRP1*.

In **chapter 4** the role of P-gp and MRP activity on treatment outcome in 104 *de novo* AML patients was evaluated. P-gp and MRP activities were determined with a flow cytometric assay, measuring rhodamine 123 accumulation with or without the P-gp inhibitor PSC833, and carboxyfluorescein accumulation with or without the MRP inhibitor MK-571, respectively. The results were compared with clinical outcome and with known prognostic factors. A low rhodamine 123 accumulation and a high rhodamine 123 efflux-blocking by PSC833 were associated with a low complete remission rate after the first cycle of chemotherapy. Patients with both low rhodamine 123 and carboxyfluorescein accumulation had the lowest complete remission rate (6%), whereas patients with both high rhodamine 123 and carboxyfluorescein accumulation had a high complete remission rate (73%) after the first chemotherapy cycle. No correlations were observed between the multidrug resistance parameters and overall survival of the AML patients. The known high risk group, including cytogenetic aberrations such as deletions of chromosome 5 or 7, and high white blood cell counts, was the only predictive parameter for the poor overall survival.

In **chapter 5**, we investigated whether P-gp or MRP activity in AML patient samples were upregulated at relapse or in refractory disease, as a result of clonal selection. Clonal selection might occur when drug resistant cell populations escape initial treatment and emerge after a certain period of time. In 30 paired samples of *de novo* and relapsed or refractory AML, no consistent upregulation of P-gp nor MRP activity was observed at relapse. P-gp activity was increased in 20% and decreased in 35% of the relapse samples, while MRP activity was increased in 35% and decreased in 15% of the relapse samples in comparison to the *de novo* samples. Since it has been described that P-gp expression is correlated with expression of the immature surface marker CD34, we also analyzed the change in surface marker expression at relapse as compared with *de novo* AML cells. A more mature phenotype was observed in 20% of the relapse cases, whereas in 25% of the cases the relapsed blasts had become less mature than the *de novo* samples. P-gp and MRP activity correlated with the phenotypic changes. Higher P-gp as well as MRP activities were observed in the relapse samples that had become less mature as compared with the primary samples. Lower P-gp and MRP activity were found when the relapse sample demonstrated a more mature phenotype than the primary sample.

A relatively new MDR protein is BCRP, also known as MXR, ABCP or ABCG2, a 655-amino-acid protein, encoded by the *BCRP* gene located on chromosome 4q22<sup>5</sup>. Overexpression of BCRP has been observed in human cancer cell lines selected for resistance with mitoxantrone, doxorubicin plus verapamil, or topotecan.

In **chapter 6**, the expression and activity were analyzed of this novel MDR protein BCRP, that might be involved in drug resistance in AML. Chemotherapeutic agents such as mitoxantrone and daunorubicin, are frequently used in the treatment of these patients, and have been described to be substrates for this drug efflux pump. In 20 paired cases of *de novo* and relapsed or refractory AML, BCRP protein appeared to be expressed at low, but variable levels. BCRP functional activity was studied by measuring the capacity of the AML cells to extrude mitoxantrone in the presence or absence of the BCRP inhibitor fumitremorgin C.

Relatively high BCRP expression in the AML blasts was correlated with a low mitoxantrone accumulation, even when the activity of P-gp and MRP were inhibited by PSC833 and MK-571. Addition of the inhibitor fumitremorgin C showed an increase in mitoxantrone accumulation only if combined with PSC833 or MK-571, in contrast to a distinct effect of fumitremorgin C alone in a BCRP overexpressing cell line. No consistent upregulation of BCRP protein expression or mitoxantrone accumulation was observed in relapsed or refractory AML samples as compared to primary cases; some cases showed an increase, other cases a decrease of BCRP protein expression at relapse. Since it has been described that BCRP is predominantly expressed in the most primitive hematopoietic side population cells, we subsequently evaluated whether a similar pattern could be observed in AML patient samples. Indeed, relatively high BCRP expression correlated with an immature immunophenotype, as determined by CD34 expression. In conclusion this study shows that BCRP protein is expressed at low, but variable levels in AML cells, especially in the immature CD34<sup>+</sup> subpopulation. BCRP was not found to be consistently upregulated in relapse or refractory AML, but was increased in the cases with a less mature immunophenotype, and decreased in the cases with a more mature immunophenotype at relapse. These findings, which were also observed for P-gp and MRP, suggest a role for the transporter proteins in maintaining a primitive phenotype in hematopoietic cells.

In conclusion, the studies presented in this thesis demonstrate that drug resistance in AML, as conferred by the expression of drug efflux pumps, is a complex phenomenon. An interplay appeared to exist between the different efflux pumps, in which a relatively

high expression of the pumps in hematopoietic cells of the normal and malignant counterpart was shown to be determined by an early maturation stage of the cells.

## GENERAL DISCUSSION

In addition to P-gp, which has been shown to be an independent poor prognostic factor of clinical outcome in AML, MRP1, although to a lesser extent, appeared to play a role in MDR in AML. Legrand *et al.* have reported that MRP activity, as determined by the modulatory effect of the inhibitor probenecid on calcein efflux, is a poor prognostic factor for the achievement of complete remission. Furthermore, the simultaneous activity of P-gp and MRP were correlated with poor clinical outcome<sup>7,8</sup>. The latter finding was confirmed by our study in 104 *de novo* AML patients, in which patients with both low P-gp and MRP activity had a complete remission rate of 73%, whereas patients with both high P-gp and MRP activity had a complete remission rate of 6% after the first cycle of chemotherapy. MRP activity was measured with an assay using carboxyfluorescein as a substrate in combination with the MRP inhibitor and leukotriene D4 receptor antagonist MK-571. Although the kinetic transport properties of MRP1 and MRP2 are different<sup>9</sup>, the substrate specificities of MRP1 and MRP2 are largely similar. It is therefore reasonable to assume that, besides MRP1, MRP2 effluxes carboxyfluorescein and that MRP2 activity is also measured in this efflux assay. MRP3, in contrast to MRP1 and MRP2, has been shown to be a low-affinity transporter for glutathione conjugates<sup>10</sup>. Since carboxyfluorescein is transported independently of glutathione<sup>11</sup>, it is probably also effluxed by MRP3. It is unlikely that MRP4 or MRP5 activity is measured in the used flow cytometric assay, since it has been reported that these proteins do not transport leukotriene C4<sup>12,13</sup> and that the leukotriene D4 receptor antagonist MK-571 does not inhibit MRP5 mediated transport<sup>13</sup>. The other *MRP1* homologues, and possibly some yet unidentified MRPs, might contribute to the carboxyfluorescein efflux assay. For that reason the assay most likely measures an overall MRP activity, instead of MRP1 activity specifically. However, we have observed a correlation between carboxyfluorescein efflux-blocking by MK-571 and MRP1 protein expression<sup>14</sup> and, to a lesser extent MRP1 mRNA expression<sup>15</sup>. These findings suggest that MRP1 fulfils a dominant role in the efflux assay.